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A. M. Madl

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PROVISIONAL SPECIFICATION

for the invention entitled:

"NOVEL PROTEINS, THEIR DERIVATIVES, HOMOLOGUES AND ANALOGUES AND USES THEREFOR AND NUCLEIC ACIDS ENCODING SAME"

The invention is described in the following statement:

NOVEL PROTEINS, THEIR DERIVATIVES, HOMOLOGUES AND ANALOGUES AND USES THEREFÖR AND NUCLEIC ACIDS ENCODING SAME

FIELD OF THE INVENTION

The present invention relates generally to amino acid sequences obtainable from SOCS proteins and which are capable of interacting with intracellular molecules. The present invention further relates to nucleic acid molecuels encoding said amino acid sequences. The amino acid sequences and the nucleic acid molecules encoding same of the present invention are useful in modulating degradation of proteinaceous molecules such as but not limited to SOCS proteins and proteinaceous molecules associated therewith.

BACKGROUND OF THE INVENTION

Bibliographic details of the publications numerically referred to in this specification are collected at the end of the description.

Cells continually monitor their environment in order to modulate physiological and biochemcial processes which in turn affects future behaviour. Frequently, a cell's initial interaction with its surroundings occurs *via* receptors expressed on the plasma membrane. Activiation of these receptors, whether through binding endogenous ligands (such as cytokines) or exogenous ligands (such as antigens), triggers a biochemical cascade from the membrane through the cytoplasm to the nucleus.

Of the endogenous ligands, cytokines represent an important and versatile group. However, of particular importance are molecules which regulate cytokine function. An example of this class of molecules are members of the family of suppressors of cytokine signalling (SOCS).

SOCS proteins contain a central SH2 domain and a C-terminal homology domain we have termed the SOCS box (1,2). The first member of this family was called CIS (cytokine-

inducible SH2-containing protein) (3) and was shown to inhibit erythropoietin and interleukin-3 receptor signalling. The inventors cloned SOCS-1 from a retroviral expression library as a cDNA whose constitutive expression inhibited interleukin-6-induced differentiation of M1 cells (1) and it was simultaneously cloned as a protein that interacted with activated JAK kinases (JAK-binding protein, JAB) (4) and as a protein with antigenic similarity to STATs (STAT-inducible STAT inhibitor, SSI) (5). The sequence similarity of SOCS-1 and CIS led to the identification of six additional members of this family (SOCS-2-7) each with an SH2 domain and a C-terminal SOCS box (2,6,7). An additional twelve proteins have been described that contain a C-terminal SOCS box but instead of an SH2 domain they contain different protein-protein interaction domains including WD40, ankyrin repeats, SPRY or small GTPase domains (2).

Following binding to their receptors, many cytokines activate receptor-associated cytoplasmic kinases called JAKs which in turn phosphorylate the receptor cytoplasmic domain and associated signal transducers and activators of transcription (STATs). Phosphorylated STAT dimers translocate to the nucleus and activate transcription of specific genes including those of CIS and some of the SOCS. SOCS proteins then recognize activated signalling molecules (including JAKs and cytokine receptors) through their SH2 and N-terminal domains and inhibit their activity (8,9). Exactly how SOCS proteins inhibit JAK kinase activity and the role of the conserved SOCS box are currently unknown.

In accordance with the present invention, the inventors have shown that the SOCS box interacts with elongins B and C and through them with the proteasome complex. Targeting of SOCS proteins and their bound activated signalling molecules to the protein degradation pathway explain how SOCS proteins simultaneously terminate a cytokine stimulation cycle and their own inhibitory action so that cells may respond to a second round of stimulation.

SUMMARY OF THE INVENTION

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a

stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

One aspect of the present invention contemplates a nucleic acid molecule encoding or complementary to a sequence encoding an amino acid sequence which is capable of interacting with elongin B and elongin C.

Another aspect of the present invention provides a nucleic acid molecule encoding or complementary to a sequence encoding an amino acid sequence which is capable of interacting with elongin B and elongin C or their homologues to form a complex wherein said complex facilitates degradation of said amino acid sequence and any other proteinaceous molecule associated with said amino acid sequence.

Yet another aspect of the present invention provides a nucleic acid molecule encoding the amino acid sequence:

$$X_1 X_2 X_3 X_4 X_5 X_6 X_7 X_8 X_9 X_{10} X_{11} X_{12} X_{13} X_{14} X_{15} X_{16} [X_i]_n X_{17} X_{18} X_{19} X_{20}$$
$$X_{21} X_{22} X_{23} [Xj]_n X_{24} X_{25} X_{26} X_{27} X_{28}$$

wherein: X_1 is L, I, V, M, A or P;

X_2 is any amino acid residue;

X_3 is P, T or S;

X_4 is L, I, V, M, A or P;

X_5 is any amino acid;

X_6 is any amino acid;

X_7 is L, I, V, M, A, F, Y or W;

X_8 is C, T or S;

X_9 is R, K or H;

X_{10} is any amino acid;

X_{11} is any amino acid;

X_{12} is L, I, V, M, A or P;

X_{13} is any amino acid;

X_{14} is any amino acid;

X_{15} is any amino acid;

X_{16} is L, I, V, M, A, P, G, C, T or S;

$[X_i]_n$ is a sequence of n amino acids wherein n is from 1 to 50 amino acids and wherein the sequence X_i may comprise the same or different amino acids selected from any amino acid residue;

X_{17} is L, I, V, M, A or P;

X_{18} is any amino acid;

X_{19} is any amino acid;

X_{20} L, I, V, M, A or P;

X_{21} is P;

X_{22} is L, I, V, M, A, P or G;

X_{23} is P or N;

$[X_j]_n$ is a sequence of n amino acids wherein n is from 1 to 50 amino acids and wherein the sequence X_j may comprise the same or different amino acids selected from any amino acid residue;

X_{24} is L, I, V, M, A or P;

X_{25} is any amino acid;

X_{26} is any amino acid;

X_{27} is Y or F;

X_{28} is L, I, V, M, A or P;

wherein said amino acid sequence is capable of interacting with elongin B and elongin C or their homologues to form a complex wherein said complex facilitates degradation of said amino acid sequence and any other proteinaceous molecule associated with said amino acid sequence.

Still yet another aspect of the present invention is directed to a peptide, polypeptide or protein comprising a sequence of amino acids capable of interacting with elongin B and elongin C or

their homologues to form a complex wherein said complex facilitates degradation of said amino acid sequence and any other proteinaceous molecule associated with said peptide, polypeptide or protein.

Another aspect of the present invention provides a peptide, polypeptide or protein comprising the amino acid sequence:

$$X_1\, X_2\, X_3\, X_4\, X_5\, X_6\, X_7\, X_8\, X_9\, X_{10}\, X_{11}\, X_{12}\, X_{13}\, X_{14}\, X_{15}\, X_{16}\, [X_i]_n\, X_{17}\, X_{18}\, X_{19}\, X_{20}$$
$$X_{21}\, X_{22}\, X_{23}\, [Xj]_n\, X_{24}\, X_{25}\, X_{26}\, X_{27} X_{28}$$

wherein: X_1 is L, I, V, M, A or P;
X_2 is any amino acid residue;
X_3 is P, T or S;
X_4 is L, I, V, M, A or P;
X_5 is any amino acid;
X_6 is any amino acid;
X_7 is L, I, V, M, A, F, Y or W;
X_8 is C, T or S;
X_9 is R, K or H;
X_{10} is any amino acid;
X_{11} is any amino acid;
X_{12} is L, I, V, M, A or P;
X_{13} is any amino acid;
X_{14} is any amino acid;
X_{15} is any amino acid;
X_{16} is L, I, V, M, A, P, G, C, T or S;
$[X_i]_n$ is a sequence of n amino acids wherein n is from 1 to 50 amino acids and wherein the sequence X_i may comprise the same or different amino acids selected from any amino acid residue;
X_{17} is L, I, V, M, A or P;
X_{18} is any amino acid;

X_{19} is any amino acid;

X_{20} L, I, V, M, A or P;

X_{21} is P;

X_{22} is L, I, V, M, A, P or G;

X_{23} is P or N;

$[X_j]_n$ is a sequence of n amino acids wherein n is from 1 to 50 amino acids and wherein the sequence X_j may comprise the same or different amino acids selected from any amino acid residue;

X_{24} is L, I, V, M, A or P;

X_{25} is any amino acid;

X_{26} is any amino acid;

X_{27} is Y or F;

X_{28} is L, I, V, M, A or P;

wherein said amino acid sequence is capable of interacting with elongin B and elongin C or their homologues to form a complex wherein said complex facilitates degradation of said amino acid sequence and any other proteinaceous molecule associated with said peptide, polypeptide or protein.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Purification of SOCS box-binding proteins from murine myeloid M1 cells. Panel A, SDS-PAGE (14% w/v Novex gel) analysis of affinity column eluates from GST-Sepharose column (lane 1), from GST-SOCS-1-SOCS-box-Sepharose column (lane 2), and from GST-SOCS-3-SOCS-box-Sepharose column (lane 3). The proteins were visualized by Coomassie blue staining. *Arrows* in lane 2 indicate the positions of the two protein bands excised for sequencing analysis by mass spectrometry. The molecular mass markers (in kilodaltons) are shown on the left. Panel B, Western blot analysis of the three affinity column eluates mentioned in panel A by anti-rat elongins B and C antibodies. Anti-rat elongins B and C antibodies (cross react with murine and human elongins B and C) were purchased from Santa Cruz and used as a mixture of antibodies.

Figure 2. Competition of SOCS-1 SOCS box interaction with elongin C. Biotinylated SOCS-1 SOCS box peptide was immobilized on streptavidin-agarose resin and used to affinity purify interacting proteins from M1 cellular extracts in the presence (+) or absence (-) of 80 mM competing non-biotinylated SOCS box peptides. Proteins were separated by SDS-PAGE on a 4-15% w/v acrylamide gel, and bands were visualized by silver staining. Soluble SOCS box peptides corresponding to SOCS-1, ASB-2 and WSB-2 prevented binding of elongin C to immobilized SOCS-1 SOCS box

Figure 3. Interaction of SOCS-1 with endogenous elongins B and C. Celluar extracts from M1 cells stablly expressing either full-length SOCS-1 or SOCS-1 lacking SOCS box (both proteins were N-terminally FLAG-tagged) were incubated with anti-FLAG antibody M2 resin and bound cellular proteins were eluted from the columns with FLAG peptide as described in Materials and Methods. Lanes 1-3 correspond to column eluates 3 to 5 from M1 cells expressing full-length SOCS-1 and lanes 4-6 correspond to column eluates 3 to 5 from M1 cells expressing SOCS-1 lacking SOCS box. The panels from top to bottom correspond to Western blot analyses by anti-FLAG, anti-elongin C, anti-elongin B, and a mixture of anti-elongin B and anti-elongin C, respectively.

Figure 4. Co-transfection of 293T cells with SOCS and elongins B and C.

Figure 5. Effect of LLnL on the endogenous expression of SOCS-3 protein. The murine macrophage-like J774 cells (4 x 10^7) were treated with either DMSO (0.1%) w/v or LLnL (50 μM) for 15 min and then stimulated with 100 ng/ml of murine IL-6 for the indicated times in the presence of DMSO or LLnL during stimulation. The cellular extracts were immunoprecipitated with a rabbit-anti-SCOS-3 polyclonal antiserum and immune complexes eluted from protein G-Sepharose beads were resolved by SDS-PAGE (13%w/v) under reducing conditions and analysed by Western blot using biotinylated rabbit-anti-SOCS-3.

Figure 6. Model of the interaction of SOCS box-containing proteins with elongins C and B (upper panel) and comparison with the phosphoprotein ubiquitin ligase complex (PULC) assembled by F box –containing proteins (lower panel).

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is predicated in part on the further elucidation of the function of the SOCS box in members of the SOCS family of proteins. In accordance with the present invention, it has been determined that the SOCS box mediates interaction with elongins B and C or homologues thereof which in turn directs the SOCS protein and any cytokines or other molecules associated with the cytokine to a protein degradative pathway.

The elucidation of the mechanism of action of the SOCS box permits the development of a range of molecules which are capable of modulating SOCS proteins and of molecules associated herewith. In addition, the SOCS box itself provides a means of inducing protein degradation when introduced into a target peptide, polypeptide or protein.

Accordingly, one aspect of the present invention contemplates a nucleic acid molecule encoding or complementary to a sequence encoding an amino acid sequence which is capable of interacting with elongin B and elongin C.

More particularly, the present invention provides a nucleic acid molecule encoding or complementary to a sequence encoding an amino acid sequence which is capable of interacting with elongin B and elongin C or their homologues to form a complex wherein said complex facilitates degradation of said amino acid sequence and any other proteinaceous molecule associated with said amino acid sequence.

In a particularly preferred embodiment, the present invention provides a nucleic acid molecule encoding the amino acid sequence:

$$X_1 X_2 X_3 X_4 X_5 X_6 X_7 X_8 X_9 X_{10} X_{11} X_{12} X_{13} X_{14} X_{15} X_{16} [X_i]_n X_{17} X_{18} X_{19} X_{20}$$
$$X_{21} X_{22} X_{23} [Xj]_n X_{24} X_{25} X_{26} X_{27} X_{28}$$

wherein: X_1 is L, I, V, M, A or P;

X_2 is any amino acid residue;

X_3 is P, T or S;

X_4 is L, I, V, M, A or P;

X_5 is any amino acid;

X_6 is any amino acid;

X_7 is L, I, V, M, A, F, Y or W;

X_8 is C, T or S;

X_9 is R, K or H;

X_{10} is any amino acid;

X_{11} is any amino acid;

X_{12} is L, I, V, M, A or P;

X_{13} is any amino acid;

X_{14} is any amino acid;

X_{15} is any amino acid;

X_{16} is L, I, V, M, A, P, G, C, T or S;

$[X_i]_n$ is a sequence of n amino acids wherein n is from 1 to 50 amino acids and wherein the sequence X_i may comprise the same or different amino acids selected from any amino acid residue;

X_{17} is L, I, V, M, A or P;

X_{18} is any amino acid;

X_{19} is any amino acid;

X_{20} L, I, V, M, A or P;

X_{21} is P;

X_{22} is L, I, V, M, A, P or G;

X_{23} is P or N;

$[X_j]_n$ is a sequence of n amino acids wherein n is from 1 to 50 amino acids and wherein the sequence X_j may comprise the same or different amino acids selected from any amino acid residue;

X_{24} is L, I, V, M, A or P;

X_{25} is any amino acid;

X_{26} is any amino acid;

X_{27} is Y or F;

X_{28} is L, I, V, M, A or P;

wherein said amino acid sequence is capable of interacting with elongin B and elongin C or their homologues to form a complex wherein said complex facilitates degradation of said amino acid sequence and any other proteinaceous molecule associated with said amino acid sequence.

The terms "SOCS" and "SOCS protein" are used in their broadest context. SOCS proteins are defined in International Patent Application No. PCT/AU97/00729 filed 31 October, 1997 and published 14 May, 1998 under serial number WO98/20023. This International application is incorporated herein by reference.

The amino acid sequence capable of interacting with elongins B and C is a referred to herein as the SOCS box. Reference herein for a "sox box" includes reference to mutants, derivatives, homologues, analogues and functional equivalents thereof. The nucleic acid molecule of the present invention may encode a SOCS protein comprising the elongins B and C- interacting region (ie. SOCS box) or may encode a peptide, polypeptide or protein which is heterologous to this region. The nucleic acid molecule may also encode a peptide comprising solely the elongins B and C-interacting region.

The present invention further contemplates a peptide, polypeptide or protein comprising a sequence of amino acids which is capable of interacting with elongin B and elongin C.

More particularly, the present invention is directed to a peptide, polypeptide or protein comprising a sequence of amino acids capable of interacting with elongin B and elongin C or their homologues to form a complex wherein said complex facilitates degradation of said amino acid sequence and any other proteinaceous molecule associated with said peptide, polypeptide or protein.

Still more particularly, the present invention provides a peptide, polypeptide or protein comprising the amino acid sequence:

$$X_1\, X_2\, X_3\, X_4\, X_5\, X_6\, X_7\, X_8\, X_9\, X_{10}\, X_{11}\, X_{12}\, X_{13}\, X_{14}\, X_{15}\, X_{16}\, [X_i]_n\, X_{17}\, X_{18}\, X_{19}\, X_{20}\, X_{21}\, X_{22}\, X_{23}\, [Xj]_n\, X_{24}\, X_{25}\, X_{26}\, X_{27} X_{28}$$

wherein: X_1 is L, I, V, M, A or P;

X_2 is any amino acid residue;

X_3 is P, T or S;

X_4 is L, I, V, M, A or P;

X_5 is any amino acid;

X_6 is any amino acid;

X_7 is L, I, V, M, A, F, Y or W;

X_8 is C, T or S;

X_9 is R, K or H;

X_{10} is any amino acid;

X_{11} is any amino acid;

X_{12} is L, I, V, M, A or P;

X_{13} is any amino acid;

X_{14} is any amino acid;

X_{15} is any amino acid;

X_{16} is L, I, V, M, A, P, G, C, T or S;

$[X_i]_n$ is a sequence of n amino acids wherein n is from 1 to 50 amino acids and wherein the sequence X_i may comprise the same or different amino acids selected from any amino acid residue;

X_{17} is L, I, V, M, A or P;

X_{18} is any amino acid;

X_{19} is any amino acid;

X_{20} L, I, V, M, A or P;

X_{21} is P;

X_{22} is L, I, V, M, A, P or G;

X_{23} is P or N;

$[X_j]_n$ is a sequence of n amino acids wherein n is from 1 to 50 amino acids and wherein the sequence X_j may comprise the same or different amino

acids selected from any amino acid residue;

X_{24} is L, I, V, M, A or P;

X_{25} is any amino acid;

X_{26} is any amino acid;

X_{27} is Y or F;

X_{28} is L, I, V, M, A or P;

wherein said amino acid sequence is capable of interacting with elongin B and elongin C or their homologues to form a complex wherein said complex facilitates degradation of said amino acid sequence and any other proteinaceous molecule associated with said peptide, polypeptide or protein.

The nucleic acid molecule and peptides, polypeptides and proteins of the present invention are preferably in isolated or purified form. The terms "isolated" and "purified" mean that the molecule has undergone at least one purification step away from other material. With respect to nucleic acid molecules, these are generally in the form of DNA such as cDNA or genomic DNA. The DNA may encode the same amino acid sequence as the naturally occurring elongins B and C-interacting region or a region containing one or more amino acid substitutions, deletions and/or additions. The nucleotide sequence may correspond to the genomic coding sequence (including exons and introns) or to the nucleotide sequence in cDNA form or it may carry one or more nucleotide substitutions, deletions and/or additions thereto.

Although not intending to limit the present invention to any one theory or mode of action, it is proposed that the elongins B and C interacting region corresponds to all or part of the SOCS box of SOCS proteins.

The SOCS proteins were initially defined as cytokine-inducible inhibitors of cytokine signalling and thought of as closing a classical negative feedback loop (1,4,5). Their direct linkage to the JAK/STAT signalling pathway was revealed by the observations that the expression of at least some SOCS proteins were induced by the STAT transcriptional activators, that they bound to activated JAKs and inhibited JAK kinase activity and thereby suppressed activation of STATs

and the subsequent ability of STATs to activate transcription of indicator genes (1,4,5,9).

The C-terminal homology domain common to SOCS-1 and CIS was subsequently shown to be conserved in six other SOCS proteins that each also contained a central SH2 domain. In addition, 12 other proteins were shown to contain a C-terminal SOCS box but these proteins did not contain a SH2 domain; instead they contained other protein-protein interaction domains such as WD40, ankyrin repeats, SPRY or small GTPase domains (2).

In accordance with the present invention, it has been shown that a common role of SOCS boxes from several different classes of proteins is to bind to elongins B and C. The elongin B and C complex has previously been shown to bind to elongin A to form an active transcriptional elongation complex or to the von Hippel Lindau (VHL) tumor suppressor protein (16,17). The sites on elongin A and VHL that interact with elongin C have been mapped and the consensus binding sequence (T,S,P)LXXX(C,S)XXX(LIV) is also conserved in the N-terminal half of all SOCS boxes (17).

It is proposed herein that the elongin B/C complex has two distinct roles. When bound to elongin A, it acts as a positive transcriptional regulator by increasing the activity of the RNA polymerase II elongation complex (18) but when bound to VHL it acts to suppress the accumulation of hypoxia-inducible mRNAs (19). The VHL/elongin B,C complex contains a putative E3 ubiquitin ligase (Cullin-2) that may target VHL-binding proteins to destruction by the proteasome. Cullin-2 appears to interact with elongin C (directly or indirectly) independently of subsequent association with VHL (19). Elongin B also contains a ubiquitin-like (UBL) sequence at its N-terminus (19) in common with several other proteins. One of these (RAD23) has recently been shown to interact directly with proteasomal subunit proteins (Cim3 and Cim5) through its UBL domain leading to an increase of protease activity associated with RAD23 (20). Analysis of the VHL gene in individuals with VHL disease has revealed that the interaction domain with elongin C is commonly mutated and that most affected individuals show a reduced ability of VHL to interact with elongins B and C (21-24). Similarly mutation of the UBL domain in RAD23 in yeast leads to ultraviolet light sensitivity suggesting that it plays an important regulatory role in nucleotide excision repair (20). These observations

suggest that coupling of VHL or RAD23 proteins to the proteasome is essential for the correct functioning of these proteins.

The N-terminal and SH2 domains of SOCS-1 and SOCS-3, at least, are required for recognition and binding to activated (tyrosine phosphorylated) signal transduction molecules (eg JAKs). It is proposed, in accordance with the present invention that the SOCS box brings into this complex elongins B and C and either through direct interactions of the elongin B UBL domain with the proteasome or through associated Cullin-2-induced ubiquitination of substrates and subsequent proteosomal association, the substrate and associated SOCS protein may be destroyed. In this scheme both activated signal transduction molecules and their negative regulators (SOCS proteins) would be destroyed after a cytokine activation cycle and the cell would be ready to respond again if cytokine is still present.

In overexpression studies, the SOCS box was not required to inhibit cytokine signalling (8,9). This implies that SOCS interaction with its targets is sufficient to inhibit signalling and that the role of the SOCS box interaction with elongins B and C may be primarily to terminate the inhibitory signal by destroying the SOCS protein. The present data indicate that the SOCS box confers protein instability on SOCS molecules in a proteasome-dependent manner. In situations where SOCS proteins are expressed at physiological levels, the ability to degrade SOCS-associated signalling molecules may become important in order to achieve maximal inhibition of cytokine-generated signals.

It was also noted in the present study that intact SOCS proteins bound less well to elongins B and C than did isolated SOCS box peptides (at least for SOCS-1 and SOCS-3). This may suggest that SOCS box availability for interaction with elongins is dependent on conformational changes associated with SOCS protein binding to its activated targets (eg JAKs). Given the efficiency of the proteosomal protein degradation system it may make some sense for SOCS proteins and signal transduction molecules to be destroyed only after they have interacted with their targets.

The present invention provides a mechanism for targeting such proteins to proteasomal

degradation via association of signalling molecules with the SOCS/CIS proteins followed by SOCS-box-mediated interaction with elongins B and C. Although not intending to limit the present invention to any one theory or mode of action. It is proposed that this interaction results in ubiquitination of SOCS/CIS and associated molecules (mediated by cullins) or that non-ubiquitinated proteins in the complex are delivered to the proteasome via the UBL sequence in elongin B. Another function of monoubiquitination of receptors is to target them to endocytosis and subsequent degradation by lysozymes rather than the proteasome (28). Consequently coupling of cell signalling molecules to the ubiquitination pathway can lead to termination of the signalling response in several different ways.

The present invention shows that the single conserved domain in 20 structurally diverse proteins (the SOCS box) serves to couple bound proteins to the ubiquitination or proteasomal compartments through interaction with elongins B and C. The SOCS-box-containing proteins thus form a family of adapter proteins, which terminate cell signalling by targeting critical molecules for intracellular degradation.

It is proposed in accordance with the present invention to use the SOCS box to target peptides, polypeptide or proteins for degradation. This has utility in gene therapy in which a gene is engineered with SOCS box DNA at the 3′-end. The fusion protein then has a SOCS box at the C-terminus and targets the protein for degradation. If the N-terminal end of the fusion protein interacts with gene causing disease then this would also be targeted for degradation. Examples of this include oncogenes or viral proteins.

SOCS box peptides are also contemplated herein for use in blocking degradation of proteins. In accordance with that aspect of the present invention SOCS box peptides are delivered into the cytoplasm and these bind elongins and prevent other proteins from being degraded. This is advantageous to, for example, prolonging the half-life of endogenous SOCS proteins so that cytokine signalling is reduced, such as in inflammatory or autoimmune disease.

It is further contemplated by the present invention to use the SOCS box elongin C interaction as a molecular screen to isolate small molecule inhibitors of this interaction. Again, prolonging

the half-life of SOCS proteins is one outcome. If, however, the small molecule recognized the site of elongin C, then protein turnover would be more generally inhibited, which is important in increasing the half life of proteins produced *via* gene therapy or in some cases preventing degradation of recombinant proteins produced in eukaryotic cells, where these proteins might be degraded by the proetasome pathway.

The present invention further contemplates other molecules associated with elongin B and elongin C interaction with the SOCS box and their use in modulating the function of the SOCS box, especially in mediating its own degradation following the formation of a complex with elongin B and C.

The nucleic acid molecule of the present invention may be isolated from any animal such as humans, primates, livestock animals (e.g. horses, cows, sheep, donkeys, pigs), laboratory test animals (e.g. mice, rats, rabbits, hamsters, guinea pigs), companion animals (e.g. dogs, cats) or captive wild animals (e.g. deer, foxes, kangaroos).

The terms "derivatives" or its singular form "derivative" whether in relation to a nucleic acid molecule or a protein includes parts, mutants, fragments and analogues as well as hybrid or fusion molecules and glycosylation variants. Particularly useful derivatives comprise single or multiple amino acid substitutions, deletions and/or additions to the SOCS box amino acid sequence.

Preferably, the derivatives have functional activity or alternatively act as antagonists or agonists. The present invention further extends to homologues of the SOCS box which include the functionally or structurally related molecules from different animal species. The present invention also encompasses analogues and mimetics. Mimetics include a class of molecule generally but not necessarily having a non-amino acid structure and which functionally are capable of acting in an analogous manner to the protein for which it is a mimic, in this case, a SOCS box. Mimetics may comprise a carbohydrate, aromatic ring, lipid or other complex chemical structure or may also be proteinaceous in composition. Mimetics as well as agonists and antagonists contemplated herein are conveniently located through systematic searching of

environments, such as coral, marine and freshwater river beds, flora and microorganisms. This is sometimes referred to as natural product screening. Alternatively, libraries of synthetic chemical compounds may be screened for potentially useful molecules.

The present invention further extends to a range of deletion mutants such as SOCS box molecules carrying deletion in the carboxy terminal region, the amino terminal region and in both the carboxy and amino terminal regions. Molecules are also contemplated by the present invention which encompasses only the carboxy terminal region or amino terminal region or fused to another peptide, polypeptide or protein.

As stated above, the present invention contemplates agonists and antagonists of the SOCS box. One example of an antagonist is an antisense oligonucleotide sequence. Useful oligonucleotides are those which have a nucleotide sequence complementary to at least a portion of the protein-coding or "sense" sequence of the nucleotide sequence. These anti-sense nucleotides can be used to effect the specific inhibition of gene expression. The antisense approach can cause inhibition of gene expression apparently by forming an anti-parallel duplex by complementary base pairing between the antisense construct and the targeted mRNA, presumably resulting in hybridisation arrest of translation. Ribozymes and co-suppression molecules may also be used. Antisense and other nucleic acid molecules may first need to be chemically modified to permit penetration of cell membranes and/or to increase their serum half life or otherwise make them more stable for *in vivo* administration. Antibodies may also act as either antagonists or agonists although are more useful in diagnostic applications or in the purification of SOCS box peptides. Antagonists and agonists may also be identified following natural product screening or screening of libraries of chemical compounds or may be derivatives or analogues of the SOCS molecules.

Accordingly, the present invention extends to analogues of the SOC box peptides of the present invention. Analogues may be used, for example, in the treatment or prophylaxis of cytokine mediated dysfunction such as autoimmunity, immune suppression or hyperactive immunity or other condition including but not limited to dysfunctions in the haemopoietic, endocrine, hepatic and neural systems. Dysfunctions mediated by other signal transducing elements such as

hormones or endogenous or exogenous molecules, antigens, microbes and microbial products, viruses or components thereof, ions, hormones and parasites are also contemplated by the present invention. They may also be useful in promoting degradation or inhibiting degradation.

Analogues of the proteins contemplated herein include, but are not limited to, modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecule or their analogues.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitisation, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or

alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid, contemplated herein is shown in Table 1.

TABLE 1

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-	Cpro	L-N-methylasparagine	Nmasn
carboxylate		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-	Norb	L-N-methylglutamine	Nmgln
carboxylate		L-N-methylglutamic acid	Nmglu
cyclohexylalanine		Chexa L-N-methylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug
D-threonine	Dthr	L-norleucine	Nle

D-tryptophan	Dtrp	L-norvaline	Nva
D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
D-α-methylleucine	Dmleu	α-napthylalanine	Anap
D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr
D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis

D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
L-ethylglycine	Etg	penicillamine	Pen
L-homophenylalanine	Hphe	L-α-methylalanine	Mala
L-α-methylarginine	Marg	L-α-methylasparagine	Masn
L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
L-α-methylserine	Mser	L-α-methylthreonine	Mthr
L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe

N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

Crosslinkers can be used, for example, to stabilise 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N_α-methylamino acids, introduction of double bonds between C_α and C_β atoms of amino acids and the formation of cyclic peptides or analogues by introducing covalent bonds such as forming an amide bond between the N and C termini, between two side chains or between a side chain and the N or C terminus.

These types of modifications may be important to stabilise molecules comprising a SOCS box if administered to an individual or if used as a diagnostic reagent.

Other derivatives contemplated by the present invention include a range of glycosylation variants from a completely unglycosylated molecule to a modified glycosylated molecule. Altered glycosylation patterns may result from expression of recombinant molecules in different host cells.

Expression includes transcription or translation or both.

Another aspect of the present invention contemplates a method of modulating activity of SOCS in a human, said method comprising administering to said mammal an effective amount of a molecule for a time and under conditions sufficient to increase or decrease elongin B and/or elongin C binding to a SOCS box. The molecule may be a proteinaceous molecule or a

chemical entity and may also be a derivative of SOCS or a chemical analogue or truncation mutant of SOCS.

Still a further aspect of the present invention contemplates a method of modulating levels of a SOCS protein in a cell said method comprising contacting a cell containing a SOCS gene with an effective amount of an inhibitor of elongin B- and/or elongin C- interaction with a SOCS box encoded by said SOCS gene for a time and under conditions sufficient to modulate levels of said SOCS protein.

Yet a further aspect of the present invention contemplates a method of modulating signal transduction in a cell containing a SOCS gene comprising contacting said cell with an effective amount of an inhibitor of elongin B and/or elongin C interaction with a SOCS box encoded by said SOCS gene for a time sufficient to modulate levels of SOCS protein with the cell.

The present invention is further described by the following non-limiting Examples.

EXAMPLE 1
MATERIALS AND METHODS

SOCS and Elongin Expression Vectors. The cDNAs encoding mouse SOCS-1,SOCS-3, WSB-1, SSB-1, ASB-1, have been described previously (1,2,9). Constructs in pEF-FLAG1 encoding these proteins, with or without the SOCS box, with an N- terminal FLAG epitope tag (DYKDDDDK) were generated by PCR essentially as described (1,9) (found at http:// www.wehi. edu.au/ willson vectors). DNA fragments encoding mouse elongins B and C were amplified using PCR from a 17-day embryo cDNA lambda library (Clontech ML5014t) and were expressed with N-terminal FLAG or myc (DQKLISEEDL) epitope tags, respectively, using the mammalian expression vector pEF-BOS.

Stable and Transient Transfection of Cell Lines. The murine monocytic leukemic cell line, M1, and the 293T human fibroblast cell line were maintained and transfected as described (9).

Preparation of GST and GST-SOCS Box Affinity Resins. DNA fragments encoding the SOCS boxes from mouse SOCS-1 (residues 172-212) and SOCS-3 (residues 186-225) with an N-terminal linker sequence (EGKSSGSGSESKVD) were generated by PCR and cloned into the bacterial expression vector pGEX-2T (10). The GST fusion proteins were purified by affinity chromatography on glutathione Sepharose 4B (Amersham Pharmacia Biotec) and affinity resins were prepared by covalently coupling 1 mg of purified proteins to 1 ml of NHS-activated Sepharose resin (Amersham Pharmacia Biotec). Before use, the affinity resins were washed with elution buffer (0.5% SDS, 50 mM DTT, 50 mM Tris-HCl, pH 8.0 and 150 mM NaCl) and equilibrated in lysis buffer (0.5% Nonidet P-40, 10 mM Tris-HCl, pH 7.5 and 100 mM NaCl).

Purification of SOCS Box-Binding Proteins. M1 cells (2 x 10^{10}) were lysed on ice for 30 min in 100 ml of lysis buffer supplemented with protease inhibitors (Complete Cocktail tablets; Boehringer Mannheim, Mannheim, Germany), 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM NaF. The total cell lysate was centrifuged at 15,000 rpm (SS34 rotor) for 15 min at 4°C and the clarified supernatant pre-incubated with 1 ml of GST-Sepharose resin for 2 h at 4°C. Half

the GST-Sepharose-depleted M1 cell lysate was incubated with 1 ml of GST-SOCS-1 SOCS box- and the other half with GST-SOCS-3 SOCS box-Sepharose resin for 2 h at 4°C. The affinity resins were washed with 40 ml of lysis buffer and then eluted with 8 x 0.5 ml of elution buffer. Eluates were concentrated to ~40 μl ,mixed with 15 μl of 4 x SDS sample buffer containing 0.4 M DTT and resolved on a 14% polyacrylamide gel (Novex). The gel was stained for 5 min with 0.1% Coomassie blue in 50% methanol and destained in 12% methanol and 7% acetic acid.

Protein Identification by Peptide Mass Fingerprinting. Protein bands were excised from the Coomassie blue-stained gel and *in-situ* tryptic digestion was performed as described previously (11). An electrospray ion trap mass spectrometer (LCQ Finnigan MAT, San Jose, CA) coupled on-line to a capillary HPLC (12,13) was used for peptide sequencing. The sequences of individual peptides were identified manually or by using the SEQUEST algorithm (incorporated into the Finnigan-MAT BIOWORKS™ software) to correlate the collision-induced dissociation spectra with amino acid sequences in the OWL protein database (14).

Peptide synthesis and biotinylation. Peptide fragments of murine SOCS-1, WSB-2 and ASB-2 corresponding to the SOCS boxes and five upstream N-terminal residues (2) were synthesized according to the *in situ* neutralization/HBTU activation protocol for Boc solid phase chemistry (15), purified using reverse phase HPLC and the products characterized by electrospray mass spectrometry. A sample of the SOCS-1 SOCS box peptide was post-synthetically biotinylated by treatment with sulfosuccinimidobiotin. Prior to biotinylation, the sidechain of the unique cysteine residue was temporarily protected by oxidation to the peptide disulfide dimer. The SOCS-1 SOCS box peptide contains no lysine residues, thus excess biotinylation reagent was used to completely and specifically modify the the amino terminus. Following biotinylation, the peptide was reduced by treatment with 5 mM DTT. Typically, peptide was coupled to streptavidin-agarose resin (Pierce immunopure; 1-2 mg streptavidin/mL resin) by incubating equal volumes of resin and 1 mg/mL peptide for 1 h, followed by extensive washing.

Competition of SOCS 1 SOCS box/elongin C interaction. M1 cells were lysed as previously described, except at a concentration of 10^9 cells/mL of lysis buffer. Streptavidin-agarose binding proteins were precleared from lysate by treating overnight at 4°C with streptavidin-agarose resin (100 µL of resin/1 mL lysate). SOCS box peptides (SOCS-1, ASB-2 and WSB-2) were solubilized in water at 10 mg/mL, and aliquots of these, or water alone, were added to 350 µL fractions of cleared lysate, followed by incubation for 3 h at 4°C. These lysates were then added to 30 µL of SOCS-1 SOCS box peptide resin and incubated a further 2 h at 4°C. The resin was extensively washed with lysis buffer and bound proteins were eluted with 20 µL of 4x SDS sample buffer. Proteins were separated by SDS-PAGE on a 4-15% reducing gel.

Detection of SOCS-1 Interaction with Endogenous Elongins. Two litres of M1 cells stably expressing either full-length SOCS-1 or SOCS-1 lacking SOCS box (with N-terminal FLAG epitopes) were grown in DME containing 5% bovine calf serum, 10 µg/ml puromycin and 50 ng/ml murine IL-6. The cells were harvested and incubated in 20 ml of culture media containing 10 µM proteasome-specific inhibitor, *N*-acetyl-L-leucinyl-L-leucinyl-norleucinal (LLnL; Sigma, St. Louis, MO) for 30 min at 37°C. The cells were lysed in 14 ml of lysis buffer supplemented with protease inhibitors (Complete Cocktail tablets), 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF and 10 µM LLnL. Total cell lysates were centrifuged at 15,000 rpm (SS34 rotor) for 15 min at 4°C and the clarified supernatants incubated with 0.3 ml of M2 anti-FLAG antibody resin for 3 h at 4°C. Resin was then washed with 10 ml of lysis buffer and the bound proteins were eluted with 6 x 150 µl of 100 µg/ml FLAG peptide in lysis buffer.

IL-6-Induced Expression of Endogenous SOCS-3 Protein. Mouse macrophage-like J774 cells were grown continuously in DME containing 10% bovine calf serum. The cells were washed once in PBS, twice with DME and starved for 1 h in DME containing 0.1% low-endotoxin bovine serum albumin (BSA; Sigma). The proteasome inhibitor LLnL dissolved in dimethyl sulfoxide (DMSO) or DMSO was added to the cells for 15 min and the cells then stimulated with 100 ng/ml of murine IL-6 for the indicated times.

Co-immunoprecipitation and Western analysis Proteins were immunoprecipitated with

anti-myc (9E10; WEHI) and protein A-Sepharose or anti-FLAG antibody conjugated to Sepharose (KM5-IC7;WEHI) and separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (Biorad, Hercules, CA) under reducing conditions. Proteins were then electrophoretically transferred to PVDF-Plus membranes (Micron Separations Inc. WestBorough, MA) and Western blotted as described (9).

EXAMPLE 2
RESULTS

Although the SOCS box appears to be a modular sequence motif present in at least twenty different proteins it does not appear to be required for inhibition of the JAK/STAT signalling pathway when SOCS proteins are overexpressed (8,9). The inventors reasoned that the SOCS box might play a regulatory role in targeting proteins to particular cell compartments or in controlling the in vivo half-lives of proteins which may become important considerations when SOCS proteins are expressed at physiological levels. The SOCS box domain is unlikely to be large enough to encode catalytic activity and is therefore likely to mediate such effects through protein-protein interactions. Consequently, the ability of the SOCS box to interact with cellular target proteins was investigated.

Isolated SOCS box sequences were used as affinity reagents to identify interacting proteins in cell lysates. Glutathione-S-transferase (GST) fusion proteins containing the SOCS box sequences from SOCS-1 or SOCS-3 were coupled to Sepharose beads and used as affinity resins to bind proteins from M1 cell lysates. After washing the beads, bound proteins were eluted with SDS buffer and electrophoresed on SDS-PAGE gels followed by staining with Coomassie Blue. The most prominent bands seen binding to both GST-SOCS fusion proteins but not the GST control were proteins of 15 and 18 kDa (Fig. 1). These bands were excised from the gel and digested in situ with trypsin. Tryptic peptides were separated by reverse-phase capillary HPLC and the column eluate fed directly onto a electrospray ion trap mass spectrometer. Collision-induced dissociation of the molecular ions was used to determine the amino acid sequences of the tryptic fragments and these were correlated against sequences in the OWL protein data base. The 18 kDa band generated 10 peptides that could be identified

as belonging to elongin B and the 15 kDa band generated 5 peptides that could be identified as belonging to elongin C (Table 1). Western blotting of the gels of the same eluates with antibodies against elongins B and C confirmed that both elongins were present in eluates from beads containing SOCS-1 or SOCS-3-box fusion proteins but not from control GST bands (Fig. 1).

Similar experiments using a biotinylated SOCS-1 SOCS box peptide bound to streptavidin-agarose also resulted in the identification by mass spectrometry of elongins B and C as interacting proteins in M1 cellular extracts. The specificity of this interaction was tested by pre-incubating extracts with unbiotinylated SOCS box peptides prior to addition of the imobilized SOCS-1 SOCS box peptide. As expected, unconjugated SOCS-1 SOCS box peptide competed for this interaction as did SOCS box peptides from, WSB-2 and ASB-2 suggesting that interaction with elongins B and C is a general property of the conserved SOCS box (Fig.2). Interestingly, identical results were obtained whether M1 cells were stimulated with cytokine (IL-6 or LIF) or not.

The inventors next tested the capacity of full-length or SOCS box-deleted SOCS proteins to interact with elongins B and C in M1 cells. M1 cells stably transfected with vectors encoding N-terminally FLAG-tagged full-length SOCS-1 or SOCS-1 lacking a SOCS box (SOCS-1/ΔSB) were lysed, the FLAG-tagged proteins were immunoprecipitated with anti-FLAG M2 antibody beads and the beads were eluted with FLAG peptide. The eluates were electrophoresed on SDS-PAGE gels, transferred to PVDF membranes and Western blotted with anti- FLAG antibodies or antibodies to elongins B and C (Fig. 3). Although full-length SOCS-1 and SOCS-1/ΔSB were expressed at similar levels, only the full-length SOCS-1 protein was associated with bound elongins B and C.

To further confirm the generality of this interaction for other proteins containing a SOCS box, 293T fibroblasts were transfected with N-terminally FLAG-tagged WSB-2 or SSB-1 along with elongin B containing a FLAG epitope or elongin C containing a myc epitope. When elongin C was immunoprecipitated with anti-myc antibodies and the electrophoresed eluates Western blotted with anti-FLAG antibodies, both WSB-2 and SSB-1 were found to co-

immunoprecipitate along with elongin B with elongin C (Fig. 4). As with SOCS-1, the interaction of elongins B and C with SSB-1 was dependent on the SOCS box as a truncated form lacking only the SOCS box failed to co-immunoprecipitate with the elongins (Fig. 4).

Because elongins B and C have been proposed to target proteins to proteasomal destruction (16, 17), the inventors tested whether endogenous SOCS proteins are degraded through the proteasomal complex. When the J774 macrophage cell line was stimulated with IL-6, SOCS-3 protein expression was elevated by 30 min, peaked at 60 min and was significantly depleted by 120 and 180 min. In contrast, cells incubated with the proteasomal inhibitor LLnL and stimulated with IL-6 showed a continual increase in SOCS-3 protein levels from 30-180 min (Fig.5) suggesting that the proteasomal complex plays a major role in rapidly degrading SOCS-3 after its induction.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

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DATED this 24th day of December, 1998

The Walter and Eliza Hall Institute of Medical Research

By DAVIES COLLISON CAVE

Patent Attorneys for the Applicants

Table 1. Tandem mass-spectrometric characterization of elongin B as the 18 kDa band and elongin C as the 15 kDa proteins bound by SOCS box sequences

Protein	Peptide No	Experimental[(a)] MH^+(Da)	Predicted[(a)] MH^+(Da)	Sequence[(b)]	Position in Protein
18 kDa Protein	1	1161.6	1162.3	HKTTIFTDAK	10-19
	2	771.5	772.0	IVEGILK	30-36
	3	1196.3	1196.3	ESSTVFELKR	20-29
	4	927.7	928.2	RIVEGILK	29-36
	5	1664.9	1664.9	IVEGILKRPPEEQR	30-43
	6	2339.6	2339.7	HKTTIFTDAKESSTVFELKR	10-29
	7	1917.8	1918.2	TTIFTDAKESSTVFELKR	12-29
	8	3056.6	3056.3	IEPFSSPPELPDVMKPQDSGGSANEQAVQ	90-118
	9	4075.0	4075.4	ADDTFEALRIEPFSSPPELPDVMKPQDSGGSANEQAVQ	81-118
	10	1066.5	1067.4	MDVFLMIR[(c)]	1-8
15 kDa Protein	1	1213.2	1213.4	REHALTSGTIK	33-43
	2	1009.6	1010.2	EIPSHVLSK	64-72
	3	1596.5	1596.8	TYGGCEGPDAMYVK[(d)]	7-20
	4	1501.5	1501.7	LISSDGHEFIVKR	21-33
	5	1159.5	1160.4	VCMYFTYK[(d)]	73-80
	6	2213.1	2212.4	AMLSGPGQFAENETNEVNFR	44-63

Sequence determination of peptides was performed by tandem mass-spectrometry in an ESI-IT mass spectrometer. Ten tryptic peptides analysed from the 18 kDa-protein corresponded to sequences in rat elongin B(Genbank accession number L42855) and six tryptic peptides analysed from the 15 kDa-protein corresponded to sequences in rat elongin C (Genbank accession number L29259) with mass errors of 0.004 – 0.080%. These peptides covered 68.6% and 66.1% of the elongins B and C sequence, respectively.

(a) Average mass values.

(b) Amino acid sequence is given using the one-letter notation.

(c) N-terminal methionine is acetylated (+42 Da).

(d) Cysteine residue is alkylated with 4-vinyl pyridine during sample preparation (+105 Da).

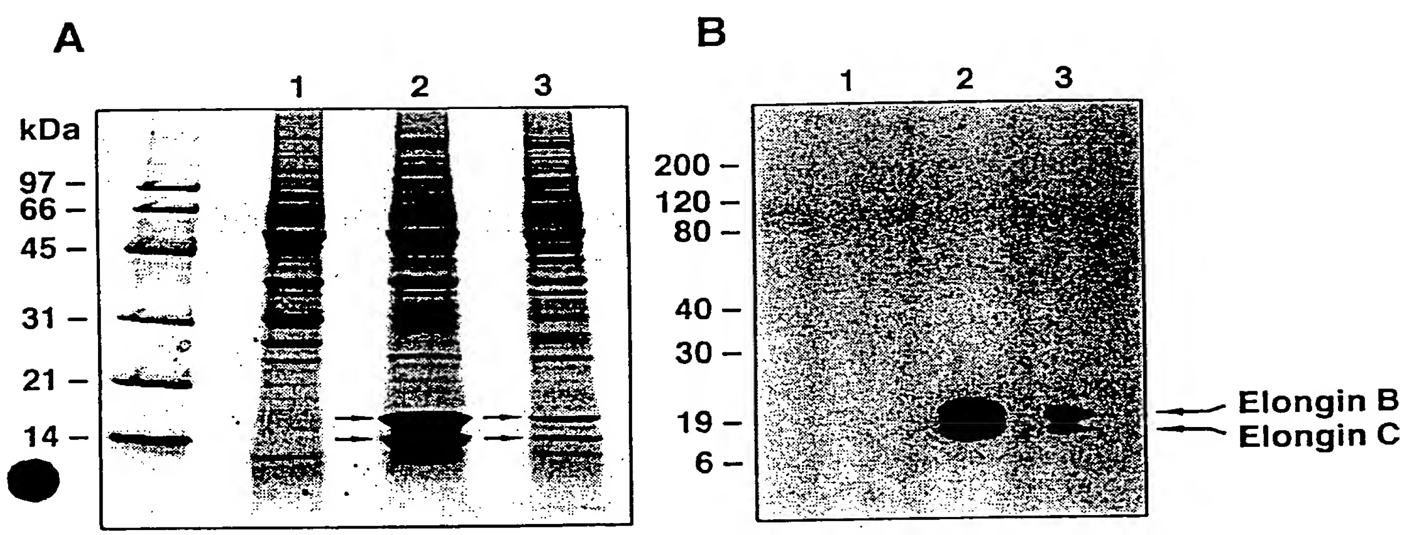

A
1
2
3
kDa
97
66
45
31
21
14
B
1
2
3
200
120
80
40
30
19
6
Elongin B
Elongin C

FIG. 1

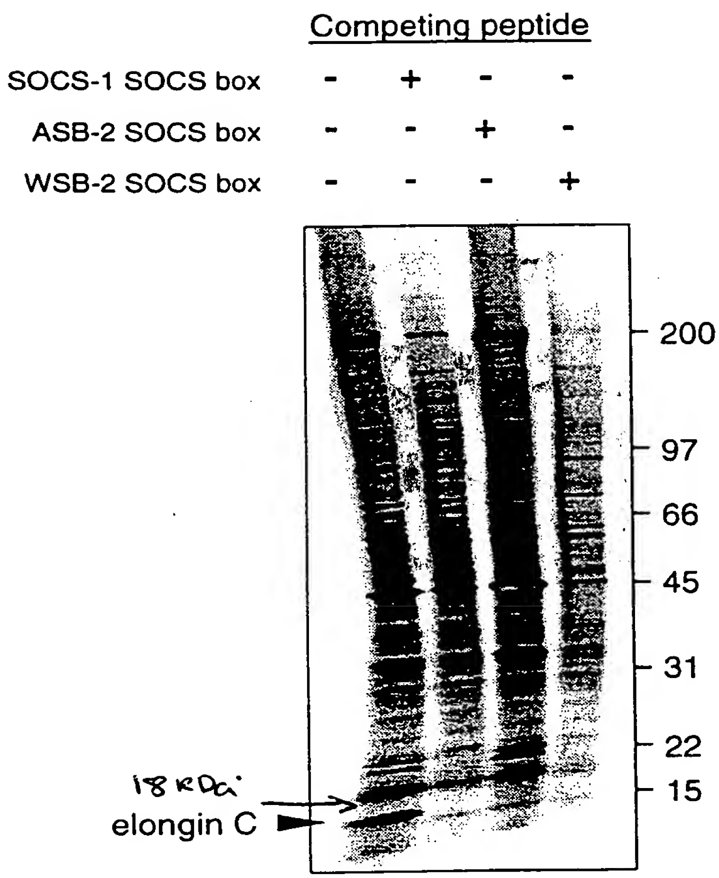

Competing peptide
SOCS-1 SOCS box - + - -
ASB-2 SOCS box - - + -
WSB-2 SOCS box - - - +
200
97
66
45
31
22
15
18 kDa
elongin C

FIG. 2

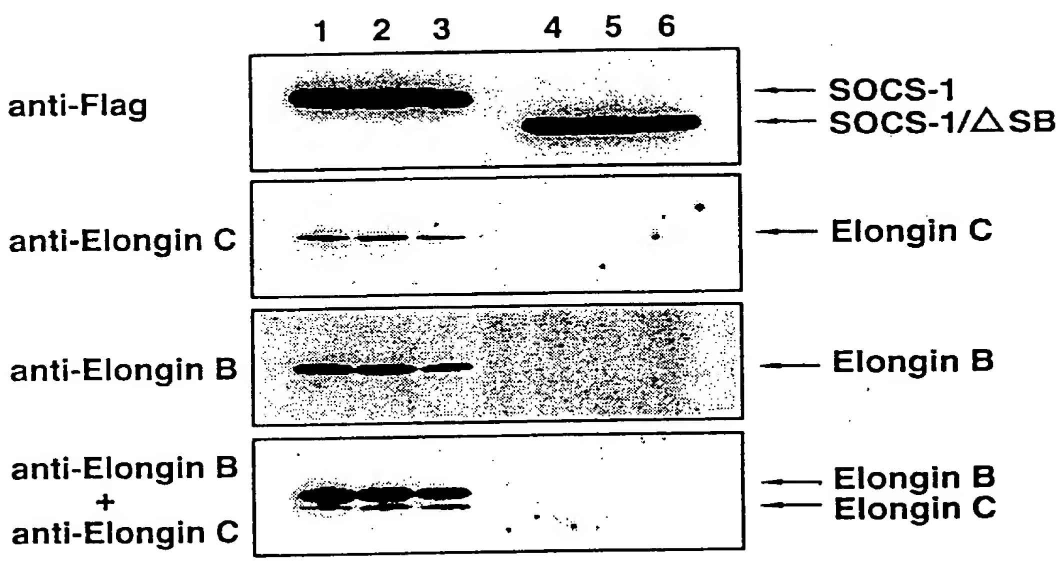

1 2 3 4 5 6
anti-Flag
SOCS-1
SOCS-1/△SB
anti-Elongin C
Elongin C
anti-Elongin B
Elongin B
anti-Elongin B
+
anti-Elongin C
Elongin B
Elongin C

FIG. 3

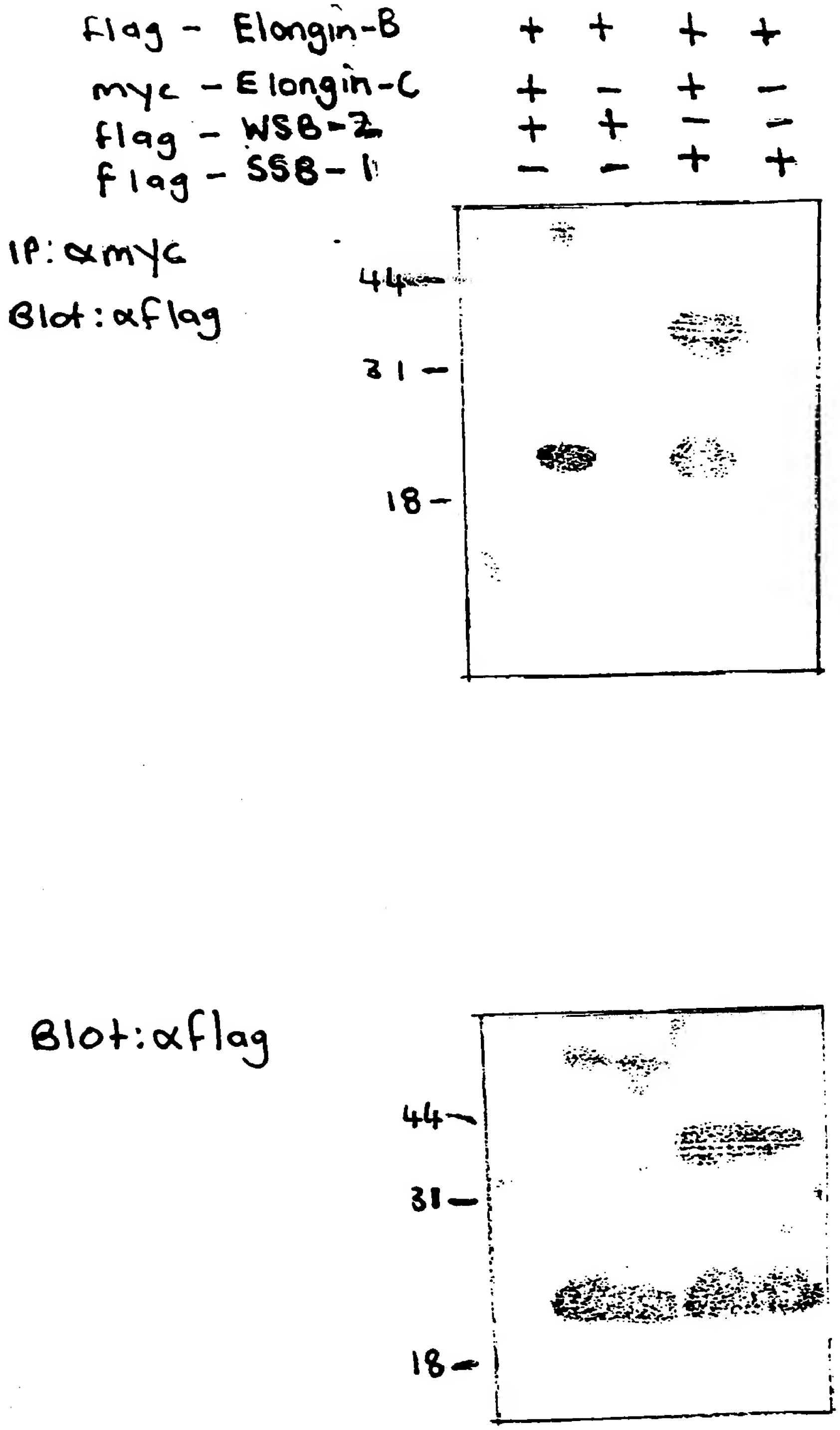

flag - Elongin-B + + + +
myc - Elongin-C + – + –
flag - WSB-2 + + – –
flag - SSB-1 – – + +
IP: αmyc
Blot: αflag
44
31
18
Blot: αflag
44
31
18

FIG. 4

981010

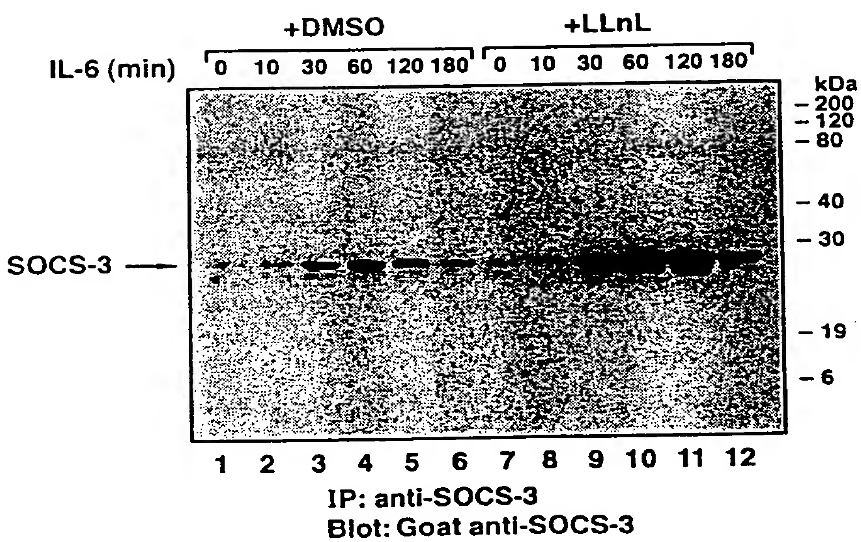
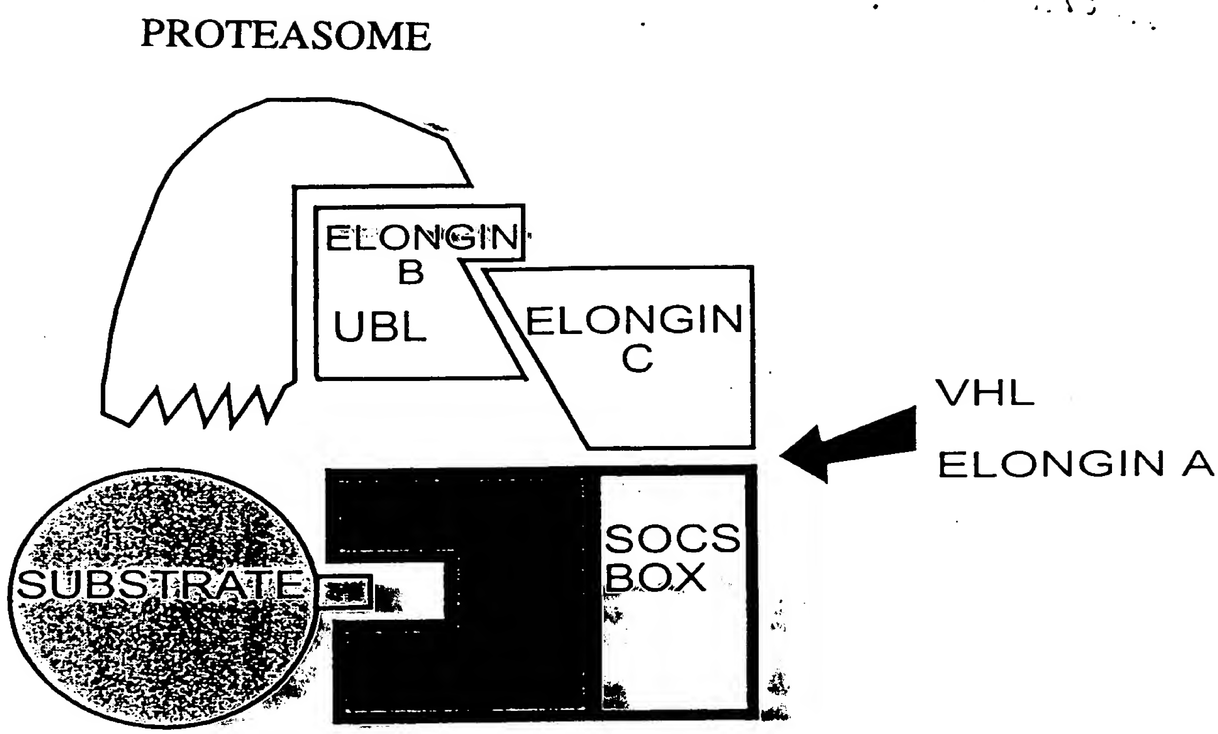

+DMSO
+LLnL
IL-6 (min)
0 10 30 60 120 180
0 10 30 60 120 180
kDa
200
120
80
40
30
19
6
SOCS-3
1 2 3 4 5 6 7 8 9 10 11 12
IP: anti-SOCS-3
Blot: Goat anti-SOCS-3

FIG. 5


PROTEASOME
ELONGIN B
UBL
ELONGIN C
VHL
ELONGIN A
SOCS BOX
SUBSTRATE

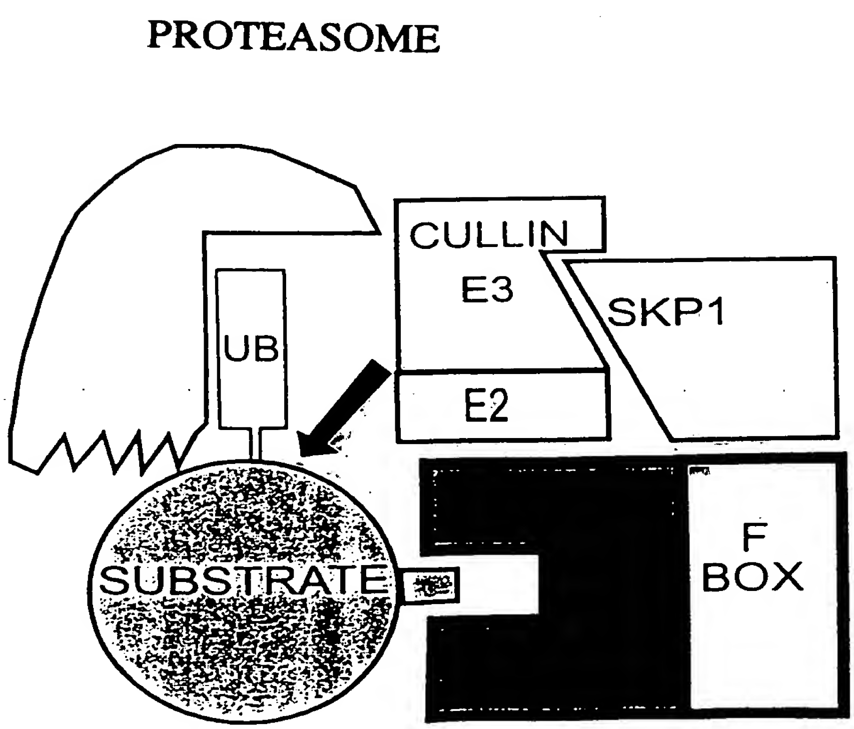

PROTEASOME
CULLIN
E3
SKP1
UB
E2
F BOX
SUBSTRATE

FIG. 6